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SEPARATION OF CAROTENOL FATTY ACID ESTERS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

FREDERICK KHACHIK* and GARY R. BEECHER
U.S. Department of Agriculture, Agricultural Research Service, Beltsville Human Nutrition Research Center, Nutrient Composition Laboratory, Building 161, BARC-East, Beltsville, MD 20705 (U.S.A.)
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SUMMARY

Employing isocratic and gradient-elution high-performance liquid chromatography (HPLC) a number of straight-chain fatty acid esters (decanoate, laurate, myristate, palmitate) of violaxanthin, auroxanthin, lutein, zeaxanthin, isozeaxanthin, and β-cryptoxanthin, prepared by partial synthesis, have been separated on a C_{18} reversed-phase column. Several chromatographic conditions were developed that separated a mixture of di-fatty acid esters (dimyristate, myristate palmitate mixed ester, dipalmitate) of violaxanthin, auroxanthin, lutein, and zeaxanthin in a single chromatographic run. Hydroxycarotenoids such as lutein, zeaxanthin, and isozeaxanthin that are not easily separated by HPLC on C_{18} reversed-phase columns, can be readily separated after derivatization with fatty acids and chromatography of their esters. Chromatographic conditions for optimum separation of carotenoids from various classes are discussed.

INTRODUCTION

The importance of generating accurate qualitative and quantitative data on carotenoids, which are among the largest naturally occurring groups of compounds found in plants, foods, and animals, has resulted in development of rugged analytical techniques that can separate, identify, and quantify these compounds[1–4]. This is primarily owing to the recent epidemiological evidence that have suggested an inverse relationship between consumption of fruits and vegetables and the risk of incidence of several types of human cancers[5]. These studies have associated carotenoids as one of the possible active ingredients in foods that their high consumption, has been correlated with reduction in cancer rates. Improvements in analytical techniques, particularly high-performance liquid chromatography (HPLC), have been important in the development of separation conditions for various classes of carotenoids isolated from food extracts. There are numerous reports on the separation of various classes of carotenoids by HPLC; however, the separation of carotenol fatty acid esters, one of the classes of carotenoids often isolated from natural products, has not received much attention. The extracts from certain fruits and vegetable (*i.e.* oranges, apricots,

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peaches, prunes, red bell peppers, squash), in which carotenoids are usually esterified with straight-chain fatty acids, are customarily saponified to remove the fatty acids and regenerate the hydroxycarotenoids[6]. Therefore, the development of a rapid HPLC method that can separate carotenol fatty acid esters within a reasonable time is required in order to assess the various carotenoids species predominant in foods as they are consumed by human beings. Among a few literature reports on HPLC of carotenol fatty acid esters is the work by Gregory *et al.*[7] and Philip and Chen[8], who recently separated the carotenol fatty acid esters in red bell peppers and Naval orange peel by HPLC. Similarly, Fisher and Kocis[9] have separated carotenol fatty acid esters of paprika pigments by HPLC. The separation of carotenoids and carotenol fatty acid esters isolated from the petals of marigold (*Tagetes erecta* L.) and primrose (*Primula vulgaris* L.) flowers have also been reported[10,11]. Recently, we reported the separation, identification, and quantification of the predominant carotenoids and carotenol fatty acid esters in extracts from several varieties of squash by HPLC[12–14]. Chromatographic conditions were developed that separated as many as 25 carotenoids as well as several of their stereoisomers, which were assigned to four classes of compounds: xanthophylls, carotenol mono-fatty acid esters, hydrocarbon carotenoids, and carotenol di-fatty acid esters.

In this report we have extended our studies on separation of carotenol fatty acid esters to a number of other carotenoids and have developed several chromatographic eluents that can be employed to separate straight-chain di-fatty acid esters (didecanoate, dilaurate, dimyristate, myristate palmitate mixed ester, dipalmitate) of some of the synthetic and common naturally occurring carotenoids (violaxanthin, auroxanthin, lutein, zeaxanthin, isozeaxanthin) by HPLC on a C_{18} reversed-phase column. The various chromatographic conditions (isocratic and gradient) described in this report readily accomplish the simultaneous separation of mixtures of carotenol fatty acid esters containing various fatty acid side chains. Finally, these conditions have been employed to separate mixtures of fatty acid esters of lutein, zeaxanthin, and isozeaxanthin, whose parent hydroxycarotenoids are not readily separated by HPLC on C_{18} reversed-phase columns.

EXPERIMENTAL*

Apparatus

A Beckman Model 114M ternary solvent delivery system equipped with a Beckman Model 421 controller was interfaced into a Hewlett-Packard 1040A rapid-scanning UV–VIS photodiode array detector. The data were stored and processed by means of a Hewlett-Packard 9000/series 300 (ChemStation) computing system which was operated with a Hewlett-Packard Model-9153B disc drive, color display monitor Model-35741, and a Model 7470A plotter. The chromatographic runs for α- and β-carotene and fatty acid esters of lutein, zeaxanthin, isozeaxanthin, and β-cryptoxanthin were monitored at 450 nm, while violaxanthin and auroxanthin fatty acid esters were monitored at 442 and 400 nm, respectively. In some cases where the

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separation of mixtures of carotenol fatty acid esters were of particular interest, the chromatographic runs were simultaneously monitored at all the above three wavelengths. The absorption spectra of the carotenoids were recorded between 200 and 600 nm as frequent as 1 scan/5 s (maximum scanning capability = 1 scan/100 ms). The HPLC detection limit for carotenol fatty acid esters was approximately 1 μg/ml.

Column

Separations were performed on a stainless-steel (25 cm × 4.6 mm I.D.) Spheri-5 RP-18 (5-μm spherical particles) column (Applied Biosystem, Analytical Division of Kratos, Ramsey, NJ, U.S.A.) which was protected with a Brownlee guard cartridge (3 cm × 4.6 mm I.D.) packed with Spheri-5 C_{18} (5-μm particle size).

Chromatographic procedures

The separations of carotenoids and carotenol fatty acid esters were carried out under three sets of HPLC conditions employing eluents A, B, and C. These eluents are described below. The retention times of various carotenol fatty acid esters (see Table I) with eluents A, B, and C were obtained by duplicate injection of each individual compound as well as injection of several mixtures of these components. These retention times which were obtained on a Spheri-5 RP-18 column were slightly different from the retention times of some of the carotenol fatty acid esters separated on a Rainin Microsorb C_{18} reversed-phase column[13]. Carotenoids and carotenol fatty acid esters were injected in the appropriate HPLC solvents (eluents A, B and C) to prevent chromatographic artifacts and HPLC peak distortion of these compounds[15]. Carotenol fatty acid esters prepared by partial synthesis were purified by thin-layer chromatography (TLC) employing eluent D. The R_F values of several of these carotenol fatty acid esters with this eluent are shown in Table II.

Eluent A (isocratic/gradient)

This eluent consisted of an isocratic mixture of methanol (10%, pump A), acetonitrile (85%, pump C), and methylene chloride–hexane (1:1) (5%, pump B) at time 0, which was followed by a gradient beginning at time 10 and completed at time 40 (min). The final composition of the gradient mixture at time 40 was: methanol (10%), acetonitrile (45%), and methylene chloride–hexane (1:1) (45%). The column flow-rate was 0.7 ml/min. At the end of the gradient the column was re-equilibrated under the initial isocratic conditions for 20 min at a flow-rate of 1.5 ml/min and finally for 5 min at 0.7 ml/min.

Eluent B (isocratic/gradient)

This eluent consisted of an isocratic mixture of methanol (15%), acetonitrile (65%), and methylene chloride–hexane (1:1) (20%) at time 0, which was followed by a gradient beginning at time 23 min and completed at time 33. The final composition of the gradient mixture was: methanol (15%), acetonitrile (40%), methylene chloride–hexane (1:1) (45%). The column flow-rate ws 0.7 ml/min. At the end of the gradient the column was re-equilibrated under the initial isocratic conditions for 20 min.

Eluent C (isocratic)

This eluent consisted of an isocratic mixture of methanol (20%), acetonitrile

TABLE I

HPLC RETENTION TIMES OF CAROTENOL FATTY ACID ESTERS SEPARATED UNDER VARIOUS CHROMATOGRAPHIC CONDITIONS EMPLOYING ELUENTS A, B OR C

Conditions are as described in the text. The chemical structures of carotenoids and carotenol fatty acid esters are shown in Fig. 1.

Fatty acid side chains	*Retention time (min)*								
	Violaxanthin (I)			*Auroxanthin (II)*			*Lutein (III)*		
	A	*B*	*C*	*A*	*B*	*C*	*A*	*B*	*C*
(A) Decanoyl (C_{10}) $R_1 = R_2 = CH_3(CH_2)_nCO$, $n = 8$	31.7	28.7	5.4	32.7	29.6	5.9	38.1	32.3	8.0
(B) Lauroyl (C_{12}) $R_1 = R_2 = CH_3(CH_2)_nCO$, $n = 10$	36.5	32.5	7.0	37.5	33.6	7.6	42.5	36.8	10.5
(C) Myristoyl (C_{14}) $R_1 = R_2 = CH_3(CH_2)_nCO$, $n = 12$	40.3	35.1	8.5	41.3	36.2	9.2	45.6	40.1	13.6
(D) Myristoyl/palmitoyl $R_1 = CH_3(CH_2)_{12}CO$ $R_2 = CH_3(CH_2)_{14}CO$	42.1	36.6	9.8	43.1	37.6	10.6	47.4	42.0	15.9
(E) Palmitoyl (C_{16}) $R_1 = R_2 = CH_3(CH_2)_nCO$, $n = 14$	43.8	38.0	11.2	44.7	39.0	12.2	49.5	44.0	19.0

* For β-cryptoxanthin, a monohydroxy carotenoid, mixed carotenol fatty acid ester is not applicable.

(40%), methylene chloride–hexane (1:1) (40%). The column flow-rate with this eluent was 0.7 ml/min.

Eluent D (isocratic)

Carotenol fatty acid esters were purified on C_{18}-reversed-phase thin-layer plates (20 × 20 cm, layer thickness 200 μm; Whatman) employing methanol (15%), acetonitrile (35%), methylene chloride (25%), and hexane (25%) as eluent.

Reagents and materials

The reference samples of (3*R*,3′*R*)-zeaxanthin, (3*R*)-β-cryptoxanthin and 15,15′-*cis*-β-carotene were provided by Hoffman-La Roche, Basel, Switzerland. Lutein was isolated from kale (*Brassica oleracea*, variety acephala) according to published procedures[2]. Isozeaxanthin was prepared from the reduction of canthaxanthin (Fluka, New York, U.S.A.) with lithium aluminium hydride[16]. The straight-chain fatty acid (C_{10}, C_{12}, C_{14}, C_{16}, and mixed C_{14}/C_{16}) esters of hydroxycarotenoids were prepared by partial synthesis from the parent hydroxycarotenoids and the corresponding fatty acid chlorides in the presence of triethylamine according to the general procedure described in text. Violaxanthin fatty acid esters were prepared by epoxidation of the corresponding zeaxanthin fatty acid esters with *m*-chloroperbenzoic acid (MCPBA) according to the general procedure described in text. Violaxanthin myristate palmitate mixed ester was similarly prepared from

Zeaxanthin (IV)			*Isozeaxanthin (V)*			*β-Cryptoxanthin (VI)*		
A	*B*	*C*	*A*	*B*	*C*	*A*	*B*	*C*
39.3	33.9	8.3	37.6	31.8	7.7	36.5	29.2	8.2
43.2	37.6	11.0	41.8	36.7	10.1	39.1	33.8	9.4
46.3	40.9	14.9	45.3	39.7	13.5	41.5	36.3	10.9
48.2	42.6	17.5	47.1	41.5	15.8		—*	
50.4	44.9	20.6	49.2	43.5	18.6	43.7	38.1	12.7

epoxidation of zeaxanthin myristate palmitate mixed ester. Auroxanthin and auroxanthin fatty acid esters were prepared from violaxanthin and violaxanthin fatty acid esters upon treatment with catalytic amount of methanolic hydrogen chloride reagent[17,18]. Free violaxanthin was obtained from saponification of violaxanthin fatty

TABLE II

R_F VALUES OF CAROTENOL FATTY ACID ESTERS SEPARATED BY THIN-LAYER CHROMATOGRAPHY EMPLOYING ELUENT D

Conditions as described in the text. The chemical structures of carotenoids are shown in Fig. 1.

Fatty acid side chains	R_F			
	Violaxanthin (I)	*Lutein (II)*	*Zeaxanthin (IV)*	*Isozeaxanthin (V)*
(C) Myristoyl (C_{14}) $R_1=R_2=CH_3(CH_2)_nCO$, $n = 12$	0.47	0.33	0.35	0.32
(D) Myristoyl/palmitoyl $R_1=CH_3(CH_2)_{12}CO$ $R_2=CH_3(CH_2)_{14}CO$	0.45	0.30	0.32	0.29
(E) Palmitoyl (C_{16}) $R_1=R_2=CH_3(CH_2)_nCO$, $n = 14$	0.43	0.27	0.29	0.26

acid esters and it was also isolated and characterized from extracts of acorn squash according to published procedures[13,14]. The reference samples of all-*trans*-α- and all-*trans*-β-carotene (Sigma, St. Louis, MO, U.S.A.) were further purified by recrystallization from methylene chloride–methanol. HPLC-grade solvents, methanol, acetonitrile, methylene chloride, and hexane (Fisher Scientific, Pittsburgh, PA, U.S.A.) were used without further purification.

General procedures for preparation of carotenol fatty acid esters by partial synthesis

Lutein fatty acid esters

A solution of an appropriate fatty acid chloride (0.0040 mmol) in dry benzene (1 ml) was added to a solution of lutein (0.0018 mmol) and triethylamine (0.054 mmol) in benzene (5 ml) and the reaction mixture was stirred under an atmosphere of nitrogen at 40°C for 20 min. The product was washed with water and 10% methanol in water and it was dried over sodium sulfate. The excess of solvent was evaporated under reduced pressure and the residue was chromatographed (eluent D). The main yellow zones (R_F = 0.22–0.33, Table II) of lutein difatty acid esters were removed and identified from their UV–VIS absorption and mass spectra. The spectroscopic evidence for structural determination of carotenol fatty acid esters have been described elsewhere[14].

Lutein myristate palmitate mixed esters. A solution of lutein in benzene was added to a mixture of myristoyl chloride and palmitoyl chloride (1:1) in benzene in the presence of triethylamine and the reaction mixture was allowed to proceed as above. The examination of the isolated product after chromatography (same as above) by HPLC revealed the presence of lutein dimyristate, lutein dipalmitate, and lutein myristate palmitate mixed esters. Under the various chromatographic conditions employed (eluents A, B and C), the two possible regio-isomers of lutein myristate palmitate mixed esters, β,ε-carotene-3-monol monomyristate-3′-monol mono-palmitate and β,ε-carotene-3-monol monopalmitate-3′-monol monomyristate, were not resolved.

Zeaxanthin, isozeaxanthin and β-cryptoxanthin fatty acid esters

The various fatty acid esters of zeaxanthin, isozeaxanthin, and β-cryptoxanthin were similarly prepared by partial synthesis from their parent compounds and purified by chromatography (eluent D) according to the procedure described above for the preparation of lutein di-fatty acid esters.

Zeaxanthin and isozeaxanthin myristate palmitate mixed esters were prepared similar to the procedure described for lutein mixed esters.

Violaxanthin fatty acid esters

A solution of each of the zeaxanthin fatty acid esters (0.0018 mmol) in hexane was allowed to react with *m*-chloroperbenzoic acid (0.0040 mmol) under an atmosphere of nitrogen at room temperature for 4 h and the course of the reaction was followed by TLC. (eluent D). After work-up, violaxanthin fatty acid esters were purified by chromatography (eluent D). Violaxanthin myristate palmitate mixed ester was similarly prepared from zeaxanthin myristate palmitate mixed ester. The HPLC retention times and absorption spectra of synthetic violaxanthin fatty acid esters under

various chromatographic conditions were identical to those of authentic samples of violaxanthin fatty acid esters isolated from a variety of acorn squash (Cucurbita Pepo) grown in New Jersey, U.S.A.[13,14]. The violaxanthin fatty acid esters prepared by partial synthesis according to this procedure were presumably a mixture of configurational isomers of violaxanthin in which the fatty acid side chains may have a *cis*- or *trans*-relationship with respect to the epoxide ring. Under the chromatographic conditions employed (eluents A, B and C) these stereoisomeric violaxanthin fatty acid esters were not resolved.

The conversion of violaxanthin fatty acid esters [$\lambda_{max.}$ = 442 nm in HPLC solvents (eluents A, B and C)] to auroxanthin fatty acid esters [$\lambda_{max.}$ = 402 nm in HPLC solvents (eluents A, B and C)] was effected with catalytic amount of methanolic hydrogen chloride and resulted in a 40-nm hypsochromic shift in the absorption maximum of the former[17,18].

RESULTS AND DISCUSSION

The chemical structures of the carotenol fatty acid esters separated by HPLC are shown in Fig. 1. The absolute configuration of some of the carotenol fatty acid esters that were prepared by partial synthesis for the present study are not known with certainty. Since the reference samples of various configurational isomers of carotenoids and carotenol fatty acid esters were not available, it was not possible to evaluate the efficiency of the chromatographic systems (eluents A, B and C) that were developed in the present study for separation of optical isomers of carotenoids. The separation of configurational isomers of carotenoids has been reported by Ruttimann *et al.*[19], who elegantly developed a method for qualitative and quantitative determination of (3*R*,3′*R*)-, (3*R*,3′*S*; *meso*)- and (3*S*,3′*S*)-zeaxanthin and (3*R*,3′*R*,6′*R*)-, (3*R*,3′*S*,6′*S*)-, and (3*S*,3′*S*,6′*S*)-lutein. This method was based on the reaction of zeaxanthin and lutein isomers with (*S*)-(+)-α-(1-naphthyl)ethyl isocyanate to afford diastereomeric dicarbamates, which were separated by HPLC. However, derivatization of crotenol fatty acid esters by this method is not possible as the hydroxyl groups have already been substituted with fatty acid side chains. Alternatively, carotenol fatty acid esters may be saponified and then derivatized according to this procedure. Although the various chromatographic conditions developed in the present study may not resolve the configurational isomers of carotenol fatty acid esters, they allow the convenient separation of various naturally occurring carotenol fatty acid esters that are commonly found in fruits and vegetables. The HPLC retention times of several carotenol fatty acid esters separated by HPLC are shown in Table I. These retention times were obtained for each of the individual carotenol fatty acid esters prepared by partial synthesis as well as mixtures of C_{10}, C_{12}, C_{14}, C_{14}/C_{16}, C_{16} fatty acid esters of each carotenoid under various chromatographic conditions (eluents A, B and C). From the retention times in Table I, it is quite clear that not only the C_{10}–C_{16} carotenol fatty acid esters of each of the hydroxycarotenoids are well separated from each other, but certain mixtures of these carotenol fatty acid esters may also be separated simultaneously. This is clearly demonstrated in the HPLC profiles of a mixture of dimyristate, dipalmitate, and myristate palmitate mixed esters of violaxanthin, auroxanthin, lutein, and zeaxanthin with eluents A and C in Fig. 2. Under various chromatographic conditions employed (eluents A, B and C) on a C_{18} reversed-phase

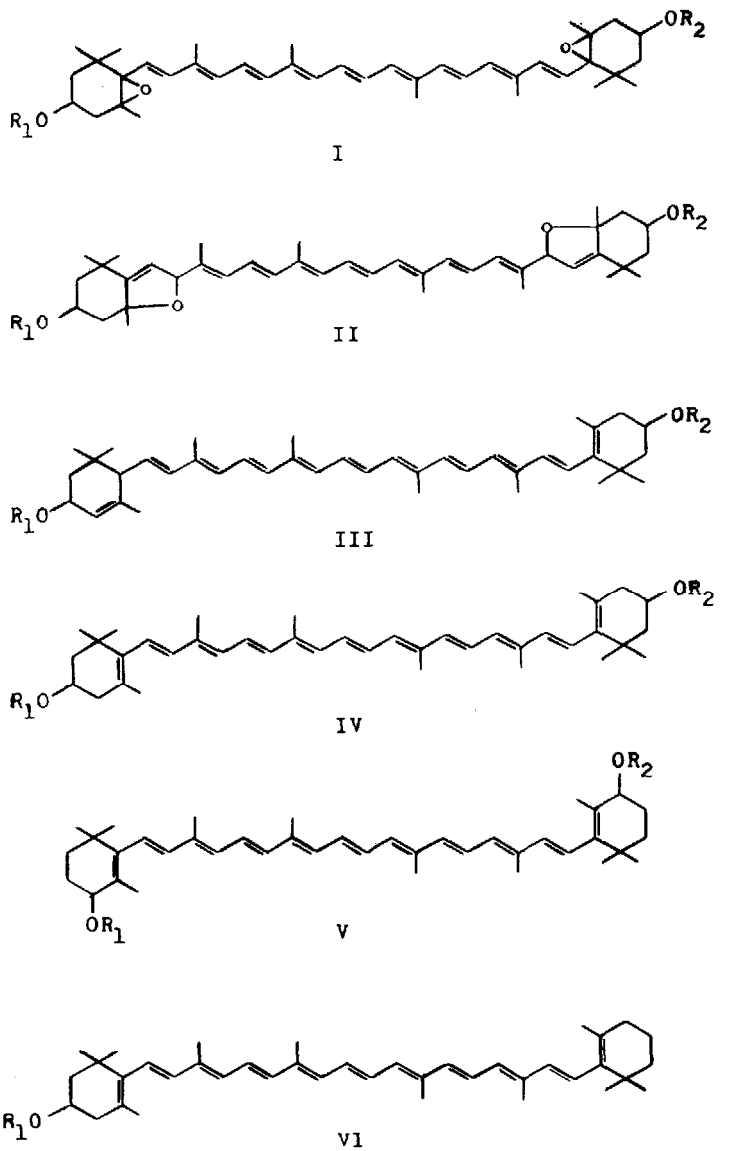

Fig. 1. The chemical structures of carotenol fatty acid esters. Where $R_1 = R_2 =$ H, I = violaxanthin, II = auroxanthin, III = lutein, IV = zeaxanthin, V = isozeaxanthin, VI = β-cryptoxanthin. The absolute configuration of these carotenoids are not shown. Where R_1 and R_2 are fatty acid esters see Table I for identification of each carotenol fatty acid ester.

HPLC column, the violaxanthin fatty acid esters are eluted first and each of the esters are then followed by auroxanthin fatty acid esters. This order of elution on C_{18} also holds for the non-esterified violaxanthin and auroxanthin[2]. The detection of violaxanthin and auroxanthin fatty acid esters in various eluents is best accomplished by monitoring the chromatographic runs at 442 and 400 nm simultaneously, as indicated in Fig. 2 by the light solid line (HPLC trace monitored at 442 nm) and the

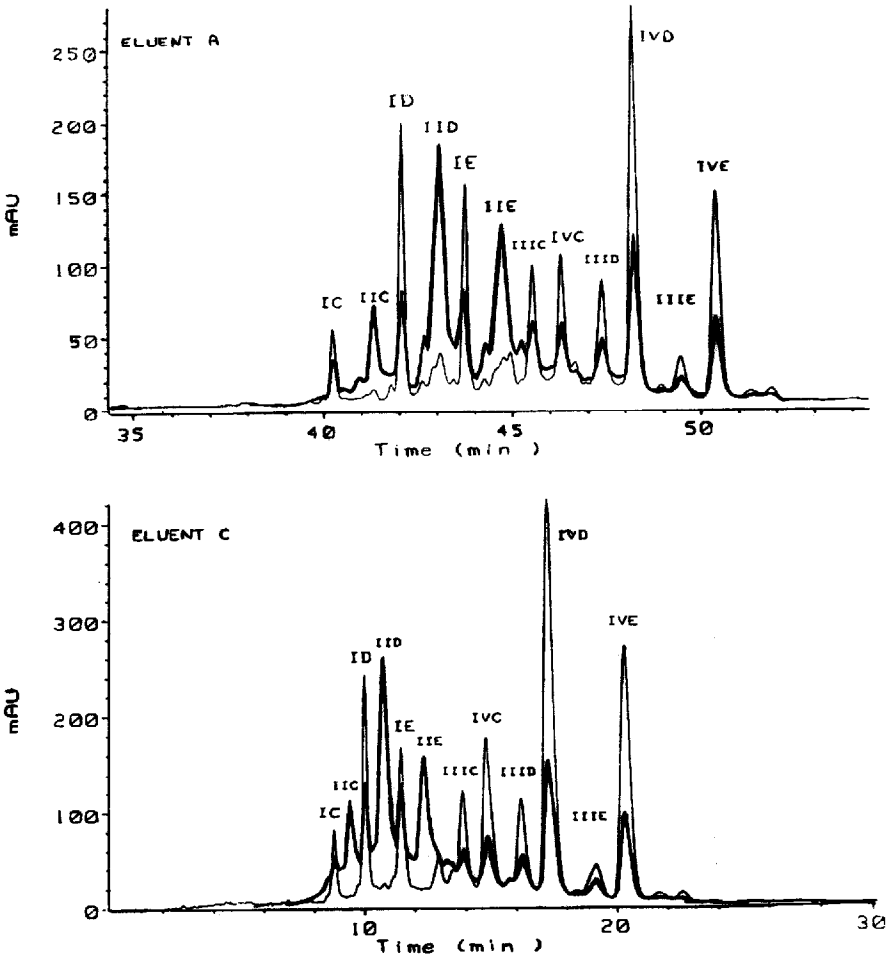

Fig. 2. HPLC profiles of a mixture of violaxanthin, auroxanthin, lutein, and zeaxanthin dimyristate, myristate palmitate mixed esters, and dipalmitate. Upper trace, eluent A; lower trace, eluent C. Chromatographic conditions and peak identification (Table I) as described in the text. Light solid lines are the HPLC traces monitored at 442 nm and dark solid lines are the HPLC traces monitored at 400 nm.

dark solid line (HPLC trace monitored at 400 nm). The rearrangement of violaxanthin fatty acid esters ($\lambda_{max.}$ = 442 nm in the HPLC solvents) induced by light, heat, or traces of acids results in the formation of auroxanthin fatty acid esters ($\lambda_{max.}$ = 402 nm in the HPLC solvents). This rearrangement is accompanied by a 40-nm hypsochromic shift in the absorption maximum of violaxanthin fatty acid esters as shown in Fig. 3 in the absorption spectra of violaxanthin and auroxanthin myristate palmitate mixed esters in the HPLC solvents.

In the order of elution on a C_{18} reversed-phase HPLC column, following violaxanthin and auroxanthin fatty acid esters, lutein and zeaxanthin fatty acid esters are eluted, respectively, with virtually no HPLC peak interference (see Fig. 2). Although the separation of the mixture of carotenol fatty acid esters described above

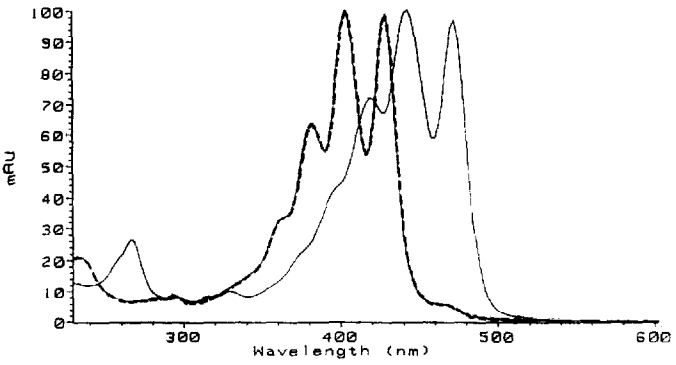

Fig. 3. Absorption spectra of violaxanthin myristate palmitate (——, $\lambda_{max.}$ = 442 nm) and auroxanthin myristate palmitate (– – – –, $\lambda_{max.}$ = 402 nm) in the HPLC solvents (eluents A or B or C). Conditions as described in the text.

may not be typical of the chromatographic profile of carotenoid extracts from natural sources, it focuses on the separation of some of the most common hydroxycarotenoids that may be esterified with common straight chain fatty acids (myristic and palmitic acid). There is no doubt that if the fatty acid esters of these carotenoids were all present in an extract from a biological source, under the chromatographic conditions employed, some HPLC peak interference between some of these esters will be inevitable. However, in most fruits and vegetables (*i.e.* oranges, apricots, peaches, prunes, and squash), where naturally occurring carotenoids are usually esterified, only fatty acid esters of selected carotenoids from each group are found to be present at one time[12].

From the retention times of carotenol fatty acid esters (Table I) within each group it seems clear that as the number of carbon atoms on the fatty acid side chains of the carotenol esters are increased the retention times of these compounds with various eluents are also increased. There seems to be no correlation between these increments and the number of the carbon atoms in the fatty acid side chains of carotenol esters. This finding is not in agreement with the observation made by Philip and Chen[8], who reported that under their chromatographic conditions, a steady increase in retention times of a number of carotenol fatty acid esters (*i.e.* β-cryptoxanthin, lutein, violaxanthin) resulted as the number of carbon atoms in the fatty acid side chains of these carotenoids were increased. At first the lack of such correlation was contributed to the lack of reproducibility of gradient chromatography, however after prolonged re-equilibriation of the HPLC column and repeated injection of the several synthetic and naturally occurring carotenol esters under various isocratic and gradient chromatographic conditions, such correlations could not be established. A possible explanation for the absence of such correlation has been provided in our earlier report on separation of carotenol fatty acid esters[13].

The chromatographic conditions developed for the separation of esterified carotenoids can also be employed to resolve selected carotenol fatty acid esters, whose parent hydroxycarotenoids are less readily separated by HPLC. The separation of a mixture of some of these carotenoids is discussed below.

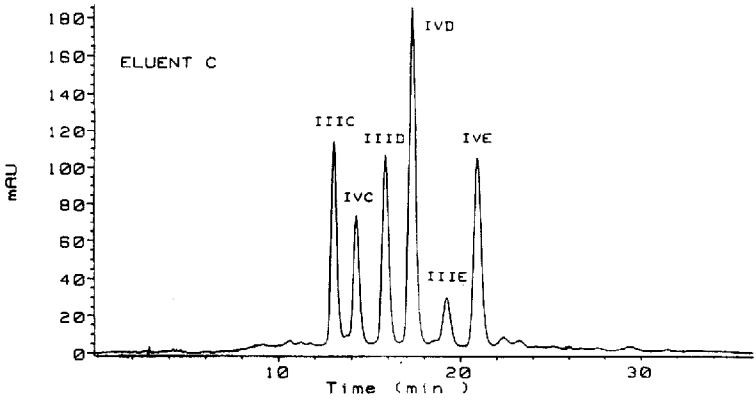

Fig. 4. HPLC profile of a mixture of lutein and zeaxanthin dimyristate, myristate palmitate mixed esters, and dipalmitate. Chromatographic conditions (eluent C) and peak identification (Table I) as described in the text.

Separation of lutein and zeaxanthin fatty acid esters

Although the non-esterified lutein and zeaxanthin can only be separated by HPLC employing eluent A, the fatty acid esters of these carotenoids are readily separated by HPLC with eluents A, B and C. A typical chromatographic profile (eluent C) of a mixture of dimyristate, myristate palmitate mixed esters, and dipalmitate esters of lutein and zeaxanthin is shown in Fig. 4. Each of the lutein fatty acid esters are eluted prior to their corresponding zeaxanthin fatty acid esters. This order of elution on C_{18} reversed-phase column also holds for the non-esterified lutein and zeaxanthin, as it will be demonstrated in the HPLC profile of a mixture of these compounds later in this text. The HPLC peak identification of a mixture such as this can be simply accomplished by comparison of the retention times of each peak in the mixture with those of the individually synthesized reference samples of these compounds. Such HPLC peak assignments can be further complemented by monitoring absorption spectra of these compounds by a rapid scanning photodiode array detector. For example, the

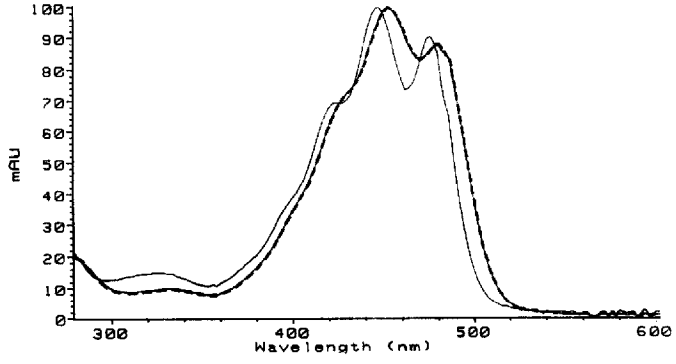

Fig. 5. Absorption spectra of lutein myristate palmitate (——, $\lambda_{max.}$ = 446 nm) and zeaxanthin myristate palmitate (– – – –, $\lambda_{max.}$ = 454 nm) mixed esters in the HPLC solvents (eluents A or B or C); conditions as described in the text.

absorption spectra of lutein fatty acid esters with a maximum at 446 nm, can be readily distinguished from the absorption spectra of zeaxanthin fatty acid esters, which have a maximum at 454 nm in the HPLC solvents. This is clearly demonstrated in the absorption spectra of lutein myristate palmitate and zeaxanthin myristate palmitate mixed esters in Fig. 5. However, in the case of carotenol fatty acid esters isolated from the extracts of natural products, additional spectroscopic evidence (*i.e.* nuclear magnetic resonance, mass spectrometry) is often necessary to establish the structure of these compounds without ambiguity. As pointed out earlier (see the experimental section) the major disadvantage of these HPLC conditions is their inability to separate the two possible regio-isomers of mixed lutein myristate palmitate. In case of the other hydroxycarotenoids studied in the present report this does not present a problem since owing to the molecular symmetry of these compounds only one mixed carotenol fatty acid ester of myristate and palmitate can be formed naturally and/or synthetically. Since the predominant carotenol fatty acid esters from natural sources are normally esterified with myristic and palmitic acid, no attempt was made in the present study to investigate the chromatographic properties of the other mixed carotenol fatty acid esters that may be prepared by random esterification of the dihydroxycarotenoids with fatty acids.

Separation of isozeaxanthin and zeaxanthin fatty acid esters

The separation of isozeaxanthin and zeaxanthin by HPLC, under chromatographic conditions employed (eluent A), only resulted in partial separation of these compounds and the HPLC peak of isozeaxanthin appeared as a trailing shoulder on that of zeaxanthin. However, the di-fatty acid esters of these hydroxycarotenoids were readily separated under various chromatographic conditions. The chromatographic profile of a mixture of isozeaxanthin and zeaxanthin didecanoate, dilaurate, dimyristate, myristate palmitate mixed esters, and dipalmitate is shown in Fig. 6. It is interesting to note that although isozeaxanthin is eluted (eluent A) after zeaxanthin on a C_{18} reversed-phase HPLC column, this order of elution is reversed for the di-fatty

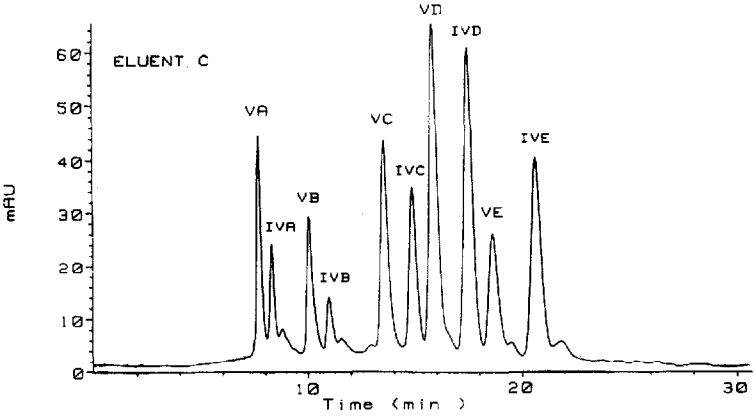

Fig. 6. HPLC profile of a mixture of isozeaxanthin and zeaxanthin didecanoate, dilaurate, dimyristate, myristate palmitate mixed esters, and dipalmitate. Chromatographic conditions (eluent C) and peak identification (Table I) as described in the text.

acid esters of these compounds under various chromatographic conditions (eluents A, B and C). The ease with which the esters of zeaxanthin (IV) and isozeaxanthin (V) are separated in comparison with their parent compounds is probably due to the fatty acid side chains in these hydroxycarotenoids that induce a more pronounced effect by these non-allylic and allylic substituents. This pronounced effect influences the parameters that affect intermolecular interactions (*i.e.* dispersion, dipole, and hydrogen bonding) between these carotenol esters and solvent molecules sufficiently different to allow the separation of these compounds.

Separation of β-cryptoxanthin fatty acid esters

Chromatographic profile of a mixture of β-cryptoxanthin decanoate, laurate, myristate, and palmitate is shown in Fig. 7. Each of the synthetic all-*trans*-β-cryptoxanthin fatty acid esters was shown to contain small amount of a mono-*cis* isomer which appeared as a shoulder following their all-*trans* isomers. These mono-*cis* isomers ($\lambda_{max.}$ = 450 nm) were tentatively identified from their UV–VIS absorption spectra, monitored by a photodiode array detector in the HPLC solvents (eluents A, B and C), which exhibited a hypsochromic shift of 4 nm from the absorption maximum of all-*trans*-β-cryptoxanthin fatty acid esters ($\lambda_{max.}$ = 454 nm). Under various chromatographic conditions employed, the HPLC peaks of the fatty acid esters of β-cryptoxanthin in most cases do not interfere with the HPLC peaks of other carotenol esters such as lutein and zeaxanthin. This is particularly important in the chromatographic evaluation of some natural extracts in which selected fatty acid esters of β-cryptoxanthin, lutein, and zeaxanthin are all present.

Optimum separation conditions for carotenoids and carotenol fatty acid esters

In the study presented in this report we described various chromatographic conditions such as eluents A, B and C that separate the fatty acid esters of several common naturally occurring carotenoids. These eluents each have a unique application in the separation of carotenoids and their related esters depending on the presence or absence of various classes of carotenoids in a given extract from natural products. In

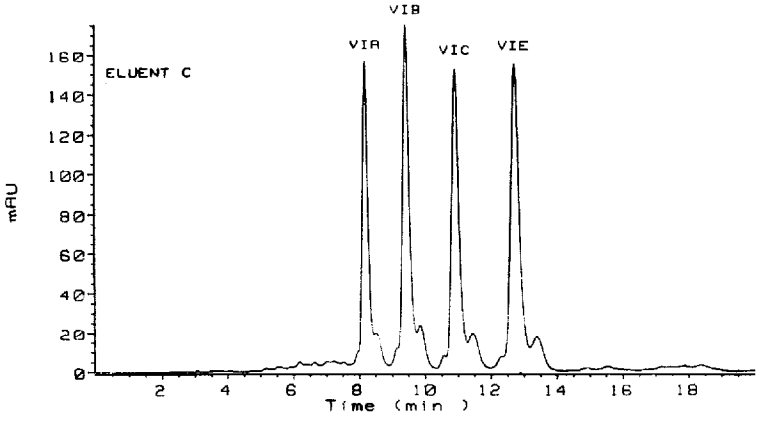

Fig. 7. HPLC profile of β-cryptoxanthin decanoate, laurate, myristate, and palmitate. Chromatographic conditions (eluent C) and peak identification (Table I) as described in the text.

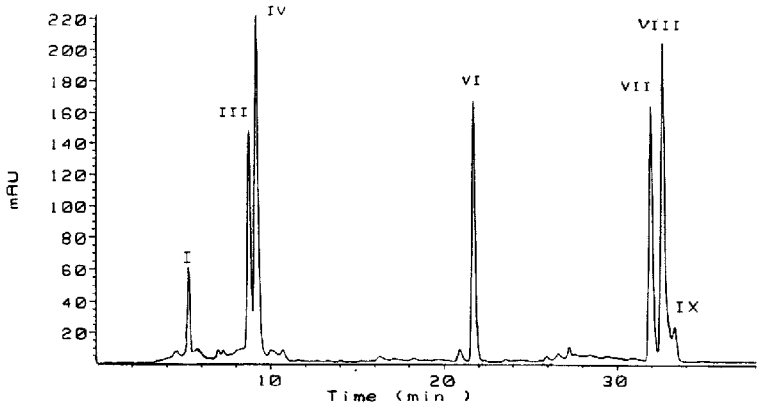

Fig. 8. HPLC profile of a mixture of violaxanthin (I), lutein (III), zeaxanthin (IV), β-cryptoxanthin (VI), all-*trans*-α-carotene (VII), all-*trans*-β-carotene (VIII), and 15,15′-*cis*-β-carotene (IX). Chromatographic conditions (eluent A) as described in the text.

an attempt to provide an insight into the application of these chromatographic conditions for an optimum separation of some of the naturally occurring carotenoids and their esters, each of these eluents are discussed below.

Eluent A. This eluent can be employed almost universally to separate a wide range of carotenoids and carotenol fatty acid esters. The chromatographic profile of a mixture of violaxanthin (I), lutein (III), zeaxanthin (IV), β-cryptoxanthin (VI), α-carotene (VII), β-carotene (VIII) and its 15,15′-*cis*-isomer (IX) is shown in Fig. 8. Under these chromatographic conditions some of the most common naturally occurring carotenoids that belong to the four classes of xanthophylls, carotenol mono-fatty acid esters, hydrocarbon carotenoids, and carotenol difatty acid esters can be separated simultaneously[13]. This eluent is particularly useful in separations in which carotenol fatty acid esters and their non-esterified parent compounds are all present in the mixture.

Eluent B. Although HPLC with this eluent has been shown to accomplish the separation of carotenol mono-fatty acid esters, hydrocarbon carotenoids, and carotenol di-fatty acid esters as efficiently as eluent A[13], it fails to separate xanthophylls such as violaxanthin, lutein, and zeaxanthin. The major advantage with this eluent is its shorter HPLC analysis time in comparison to eluent A (see Table I).

Eluent C. This eluent provides a convenient isocratic separation for carotenol di-fatty acid esters and does not separate the other classes of carotenoids. This isocratic HPLC condition is particularly important for separation and isolation of carotenol fatty acid esters on both analytical and preparative scales. The reasonably short analysis time with this eluent provides a convenient method for the HPLC analysis of extracts from natural sources in which, carotenoids are only present in the esterified forms.

Nomenclature

For convenience the trivial names of several naturally occurring carotenoids have been used throughout this text. The trivial and systematic names as well as chemical structures of these carotenoids with ε- and β-type end groups have been tabulated by Straub[20].

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REFERENCES

1 G. R. Beecher and F. Khachik, *J. Natl. Cancer Inst.*, 73 (1984) 1397–1404.
2 F. Khachik, G. R. Beecher and N. F. Whittaker, *J. Agric. Food Chem.*, 34 (1986) 603–616.
3 F. Khachik and G. R. Beecher, *J. Agric. Food Chem.*, 35 (1987) 732–738.
4 G. R. Beecher and F. Khachik, in T. Moon and M. Micozzi (Editors), *Nutrition and Cancer Prevention: The Role of Micronutrients*, Marcel Dekker, New York, 1988, Ch. 5, in press.
5 M. Micozi, in T. Moon and M. Micozzi (Editors), *Nutrition and Cancer Prevention: The Role of Micronutrients*, Marcel Dekker, New York, 1988, Ch. 3, in press.
6 G. Noga and F. Lenz, *Chromatographia*, 17 (1983) 139–142.
7 G. K. Gregory, T. S. Chen and T. Philip, *J. Food Sci.*, 52(4) (1987) 1071–1073.
8 T. Philip and T.-S. Chen, *J. Chromatogr.*, 435 (1988) 113–126.
9 C. Fisher and J. A. Kocis, *J. Agric. Food Chem.*, 35 (1987) 55–57.
10 W. Gau, H.-J. Ploschke and C. Wünsche, *J. Chromatogr.*, 262 (1983) 277–284.
11 F. Zonta, B. Stancher and G. P. Marletta, *J. Chromatogr.*, 403 (1987) 207–215.
12 F. Khachik, G. R. Beecher and W. R. Lusby, presented at the *8th International Symposium on Carotenoids, Boston, MA, July 27–31, 1987.*
13 F. Khachik and G. R. Beecher, *J. Agric. Food Chem.*, 36 (1988) in press.
14 F. Khachik, G. R. Beecher and W. R. Lusby, *J. Agric. Food Chem.*, 36 (1988) in press.
15 F. Khachik, G. R. Beecher, J. Vanderslice and G. Furrow, *Anal. Chem.*, 60 (1988) 807–811.
16 F. J. Petracek and L. Zechmeister, *J. Am. Chem. Soc.*, 78 (1956) 1427–1434.
17 C. H. Eugster and P. Karrer, *Helv. Chim. Acta*, 40 (1957) 69–79.
18 K. Tsukida and L. Zechmeister, *Arch. Biochem. Biophys.*, 74 (1958) 408–426.
19 A. Ruttimann, K. Schiedt and M. Vecchi, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 6 (1983) 612–616.
20 O. Straub, in H. Pfander (Editor), in collaboration with M. Gerspacher, M. Rychener and R. Schwabe, *Key to Carotenoids*, Birkhauser, Basel, 2nd ed., 1987.